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Serum Induced Cell Death

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ABSTRACT—In the culture system of human fetal lung fibroblasts (TIG-1) using Eagle's MEM containing various proportion of fetal bovine serum (FBS), FBS stimulated cell growth within the range below 40%. FBS at 60% and above inhibited cell growth in a dose-dependent manner and also induced cell death. As well as TIG-1, several types of cells presently tested also exhibited cell death in high concentration FBS. Bovine plasma also showed the same type of cytotoxicity as FBS. As a first step toward identifying the molecular basis of serum toxicity, FBS was divided into high and low molecular weight fractions by ultrafiltration and these were tested at various concentrations on variety of cell lines including TIG-1 human-fetal lung fibroblast, HeLa human epithelioid carcinoma cells, CPAE bovine aorta endothelial cell, FR Rat skin fibroblast, B16 mouse melanoma, and rat embryo primary cultures. When the macromolecular fraction was supplemented with inorganic salts and nutrients and its osmolarity was adjusted with Eagle's MEM, it showed no toxic effects over a blood range of concentrations. By contrast, a high concentration of low molecular- weight fraction induced cell death. These data suggest FBS contains low molecular-weight (<1,000) factor(s) which cause cell growth inhibition and cell death.

INTRODUCTION

When cells are cultured *in vitro*, serum is commonly added to defined basal media as a source of nutrients and macromolecules essential for growth. As serum is complicatedly constituted of numerous types of biologically active substances, it's roles in culture is also complex and difficult to clearly defined. A proposal that hormonally defined media could be substituted for serum-containing media [5] gave the concept of serum-free media. During the progress of development of the serum-free media, a number of factors such as growth factors, hormones, or cell attachment factors have been identified as essential factors for the cell culture. Despite the efforts, serum is generally still the best additive and many types of cells especially normal cells can survive for long period only with serum, implying the existence of other unknown factors in serum. Some of possible function of serum were postulated [4, 5]. Ever since Carrel and his co-workers reported in 1920's that sera from adult animals inhibit cell growth [1, 2], serum from young animals especially fetal bovine serum (FBS) has been most commonly used for cultures. Adult sera are known to contain much more immunoglobulins, complement, hormones, and lipids at higher concentrations than those from fetal and early animals, and these are thought to inhibit growth. In addition, some reports suggest that neoplastic transformation and chromosomal abnormalities occur less frequently in medium containing FBS as opposed to horse serum [3]. Nevertheless it is well known that FBS occasionally shows cytotoxicity [6], the cause of which is unclear. Since there are relatively few studies of this phenomenon, we began an investigation of its molecular basis. Initially, we found that cells cultured in high concentration of FBS appended to undergo cell death. When the macromolecular fraction (>M.W. 1,000) was separated from FBS by ultrafiltration and the low molecular weight fraction was substituted by Eagle's MEM, this macromolecular FBS stimulated growth of TIG-1 human fetal lung fibroblast and some other cell types of cultures in a dose-depended manner without causing cell death. By contrast the low molecular weight fraction (<M.W. 1,000) caused

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cell death of TIG-1 cell. These results suggested that the FBS low molecular weight fraction contained cytotoxic factor(s). Serum, a product of blood coagulation, is formed at sites of tissue injury *in vivo* and may contain many factors in addition to those in plasma. Thus we also conducted a comparative experiment between serum and plasma in cytotoxicity, showing no practical difference.

MATERIALS AND METHODS

Cell cultures

Human fetal lung fibroblasts TIG-1, human epithelioid carcinoma HeLa, mouse melanoma B16 were obtained from JCRB. Mouse fetal skin FR was from ATCC. All cells were maintained in Eagle's MEM containing 10% FBS. Cell cultures were observed and photographed using Nicon Diaphot phase-contrast inverted microscope. Rat whole-embryo primary culture (RWEC) was established as follows. Wistar-lamichi rat (Japan Crea co.) embryos at 16 days of age were collected and minced in 60 mm petri-dishes. After digestion in 0.1% trypsin, 0.02% EDTA CM-PBS at 37°C for 30 min, the tissue was minced again and dispersed in MEM+10% FBS. Undigested tissue clumps were removed with a nylon-mesh, and cells were collected by centrifugation. Cells were seeded in a 35 mm petri-dish/one embryo (Corning) in MEM+10% FBS, and cultures were washed to remove cell debris and red blood cell. Medium was changed everyday, and when the cultures reached confluence, the cells were harvested and used for experiments.

Serum

We preliminarily tested 14 lots of obtained from several commercial sources (Hyclone, Salmond Smith Biolab Ltd., GIBCO BRL, Boheringer Mannheim, Bio cell, IRVINE) with TIG-1. All showed almost identical cytotoxicity. Heat treatment (57°C, 30 min) had no effect on serum toxicity. Accordingly, we used FBS from Boheringer mannheim (Lot 562044) for all experiments without heat treatment.

Medium

Eagle's MEM "Nissui" (Nissui Pharmaceutical Co., Ltd.) was used as the basal medium for all the experiments. Hanks solution "Nissui" (Nissui Pharmaceutical Co., Ltd.) was used for washing the cell.

Ultrafiltration of FBS

Ultrafiltration of FBS was performed using ultrafiltration membrane YM2 (M.W. 1,000) (Amicon Co.). FBS was concentrated 10-fold and diafiltrated with a 10-fold volume of deionized water to remove the low-molecular-weight fraction. The resultant macromolecular fraction of FBS (MM-FBS) was again concentrated 10-fold then MM-FBS was diluted to the original FBS volume with 10/9 concentrated Eagle's MEM, and the pH and osmolality were adjusted to 7.2 ± 0.2 and 290 ± 10 mosmol/Kg·H_2O, as the ultrafiltrated FBS (UF-FBS). The FBS filtrate (<M.W. 1,000) was freeze-dried and dissolved in 90% volume of deionized water, and the insoluble fraction was removed by centrifugation. The pH of supernatant was adjusted to be pH 7.4 with 150 mM HCl, and MM-FBS was diluted 10-fold with this supernatant to prepare recombined FBS (RC-FBS). The osmolarity of RC-FBS was slightly higher than that of FBS (298–306 mosmol/Kg·H_2O). The ultrafiltrate, low molecular fraction of FBS, was adjusted to pH 7.4 with 0.15 M HCl, almost equivalent to FBS in osmotic-pressure. This prepared ultrafiltrate was used as medium for culturing cells.

Preparation of plasma

Fresh bovine carotidal blood obtained from a local slaughterhouse was immediately added with sodium citrate (final conc. 0.5%) and kept at 15°C. The blood was centrifuged at 6,800 g from 30 min in a continuous centrifuge to remove the hemocyte fraction. The supernatant was stepwise microfiltered and finally passed through a 0.22 μm filter. After the keeping at 4°C for about 1 week, the supernatant was divided into three fractions and processed just before experiments as follows: 1. fibrin was simply spinned out (crude plasma), 2. treated with 1% of 100 unit/ml thrombin (Sigma) at 37°C for 16 hr and contrifuged (thrombin-

treated plasma), and 3. incubated at 37°C for 16 hr without thrombin treatment and centrifuged (incubated plasma).

Measurement of Serum effect

Cells were harvested from confluent 25 cm^2-culture flasks (Corning) after treatment with 0.25% trypsin and 0.02% EDTA in CMF-PBS. An appropriate number of cells in 100 μl/well MEM+10% FBS were seeded into 96 well culture plates (Corning). The cell number used resulted in 1/20–1/5 confluence in each well, and they were as follows; TIG-1 4×10^3/well, CPAE 3×10^3/well, FR 4×10^3/well, HeLa 3×10^3/well, B16 6×10^3/well, RWEC 6×10^3/well, and 6×10^4/well.

After overnight incubation at 37°C in 5% CO_2, the culture medium was removed. The wells were washed with Hanks balanced solution and 100 μl of test medium was added. After 6 days of culture, the cell number in each well was determined using a Coulter Counter ZM (Coulter Electronics).

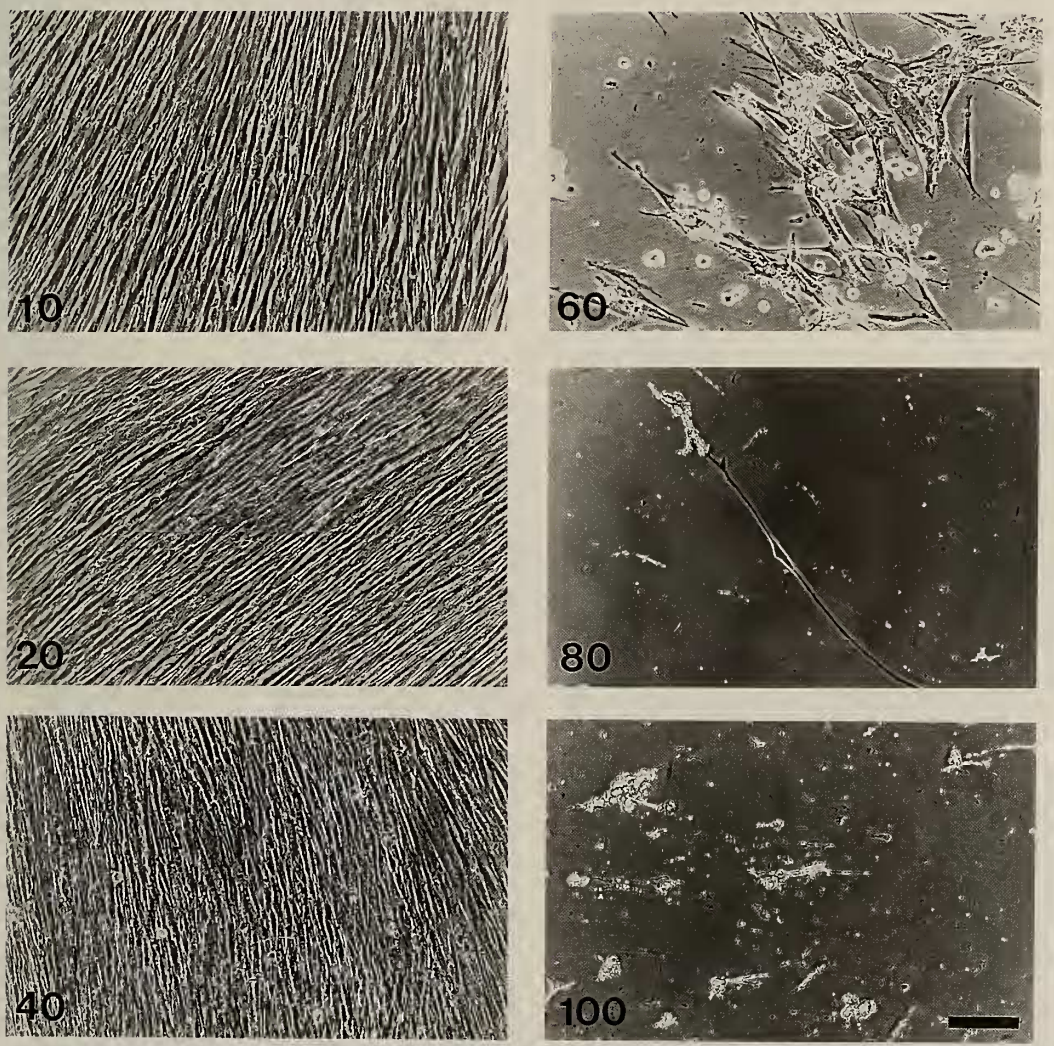

FIG. 1. Effect of FBS on TIG-1 human fetal lung fibroblasts. Human fetal lung fibroblast TIG-1 cells were seeded at 1.0×10^4 cells/well in a 24-well plate, and after 24 hr incubation at 37°C the medium was changed to MEM containing the indicated concentrations of FBS. Six days later photographs were taken. Bar=100 μm.

RESULTS

TIG-1, human fetal lung fibroblasts, were cultured in Eagle's MEM containing various proportion of FBS. FBS stimulated cell growth within the range below 40%. FBS at 60% and above inhibited cell growth in a dose-dependent manner and also induced cell death (Fig. 1, 2). The time required for the cell death was 2–3 days at 60% FBS during which slight cell growth was observed. On the other hand FBS at 80% and above induced complete cell death within 24 hr accompanied with no cell growth. FBS from several different commercial sources had essentially the equal effects on TIG-1 cells. Figure 1 shows the morphologices of TIG-1 cells cultured for 6 days in media containing several different concentrations of FBS. FBS cytotoxicity was dependent upon cell density affected for the degree of the cytotoxicity of FBS. The higher density, the slower the death of the TIG-1 cells (data not shown).

In order to estimate the molecular-weight of the cytotoxic factor(s), we separated FBS into a macromolecular fraction and a low molecular weight fraction by ultrafiltration, and pH and osmolarity were then adjusted. Cytotoxicity was not retained above ultrafilter (Amicon Co.) of the following sizes: YM10 (M.W. 10,000), YM5 (M.W. 5,000), YM2 (M.W. 1,000) (data not shown). Accordingly we used ultrafilter YM2 for the experiments. UF-FBS showed no cytotoxicity and stimulated cell growth dose-dependently (Fig. 2). Recombined FBS (RC-FBS) promoted cell growth more effectively, but the cytotoxicity was comparable to intact FBS. These results indicated that the low molecular-weight fraction of FBS is necessary for the cytotoxicity.

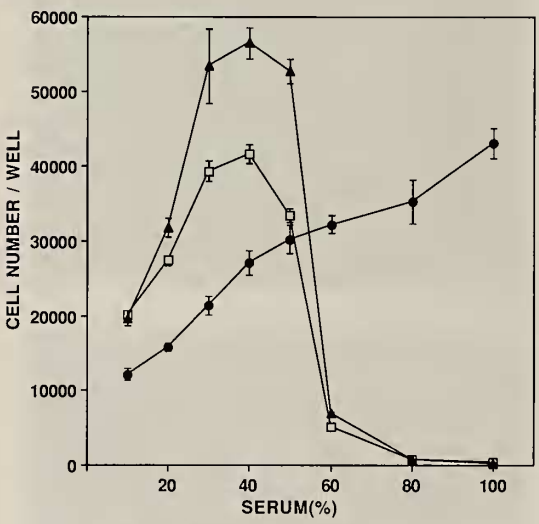

FIG. 2. Effect of serum concentration of TIG-1 cell growth. □: FBS, ●: UF-FBS, ▲: RC-FBS. Human fetal lung fibroblast TIG-1 cells were seeded at 4.0×10^3 cells/well in a 96-well plate, and after 24 hr incubation at 37°C medium was changed to MEM containing the indicated concentrations of FBS. Six days later, cell number was determined with a Coulter Counter.

To determine whether the low-molecular-weight fraction is sufficient for cytotoxicity, TIG-1 cells were cultured in various concentration of FBS-filtrate (from 10% to 100%). Cell death was maximal in 80% filtrate (Fig. 3). The FBS filtrate had cytotoxic activity, but it was slightly less than intact FBS. Thus most of the activity was able to pass through the filter.

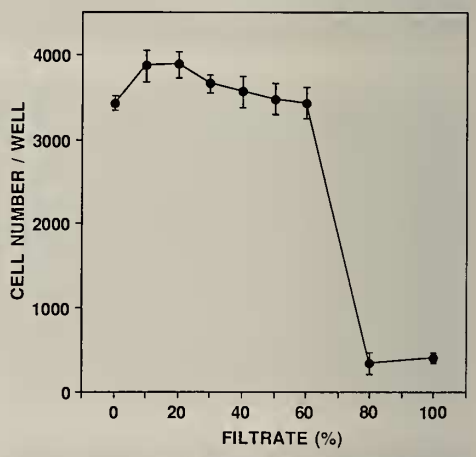

FIG. 3. Cytotoxicity assay of the FBS ultrafiltrate. Human fetal lung fibroblasts (TIG-1) were seeded at 4.0×10^3 cells/well in a 96-well plate, and after 24 hr incubation at 37°C the medium was changed to MEM containing the indicated concentrations of FBS ultrafiltrate. Six days later, cell number was determined with a Coulter Counter.

The cell death induced by FBS is not a specific phenomenon for TIG-1 human fetal lung fibrlblasts. We observed that several other types of cells also ceased to grow and died in FBS. Figure 4 shows cultures of TIG-1 and HeLa cells in media

containing FBS or UF-FBS. Figure 5 depicts the growth patterns of four types of cells cultured in media containing 10–100% of FBS. Though the cells were variously sensitive, all of the type showed cell death in FBS. Interestingly, growth of B16 mouse melanoma was inhibited by a high concentration of UF-FBS. This suggests that growth inhibitor remains in UF-FBS, although it is possible that a small amount of low molecular weight cytotoxic material did not pass completely through the filter. B16 melanoma was very sensitive to the cytotoxicity of FBS, and the cytotoxicity of the ultrafiltrate was less than FBS. In addition to TIG-1, HeLa, B16, CPAE, and FR cells, we tested human adult dermal fibroblasts (HDF) and Bovine carotid artery endothelial cells (HH). These culture also showed cell death in high concentration FBS.

It is possible all of the cell lines examined lost their resistance to cytotoxic substances during long term culture in media containing 10% FBS. We, therefore, tested rat whole-embryo primary cultures (RWEC) (Fig. 6), but the results were similar. All of the cells died in FBS. When the cells from embryos were cultured in 100% FBS, we couldn't observe any growing cells (data not shown). In the case of RWEC, cell-density was related to cell sensitivity to FBS cytotoxicity. Low density cultures were more sensitive than high density cultures. It is possible that cell-cell interactions or cell adhesion conditions affected cell tolerance to FBS cytotoxicity.

Cytotoxicity of plasma as compared with serum was studied upon cultures using medium supplemented either with the crude plasma, thrombin treated plasma or incubated plasma (Fig. 7). As the result, paractically non of three kinds of plasma was different in cytotoxicity from FBS, and TIG-1 cells also exhibited cell death when cultured with high concentration of those plasma.

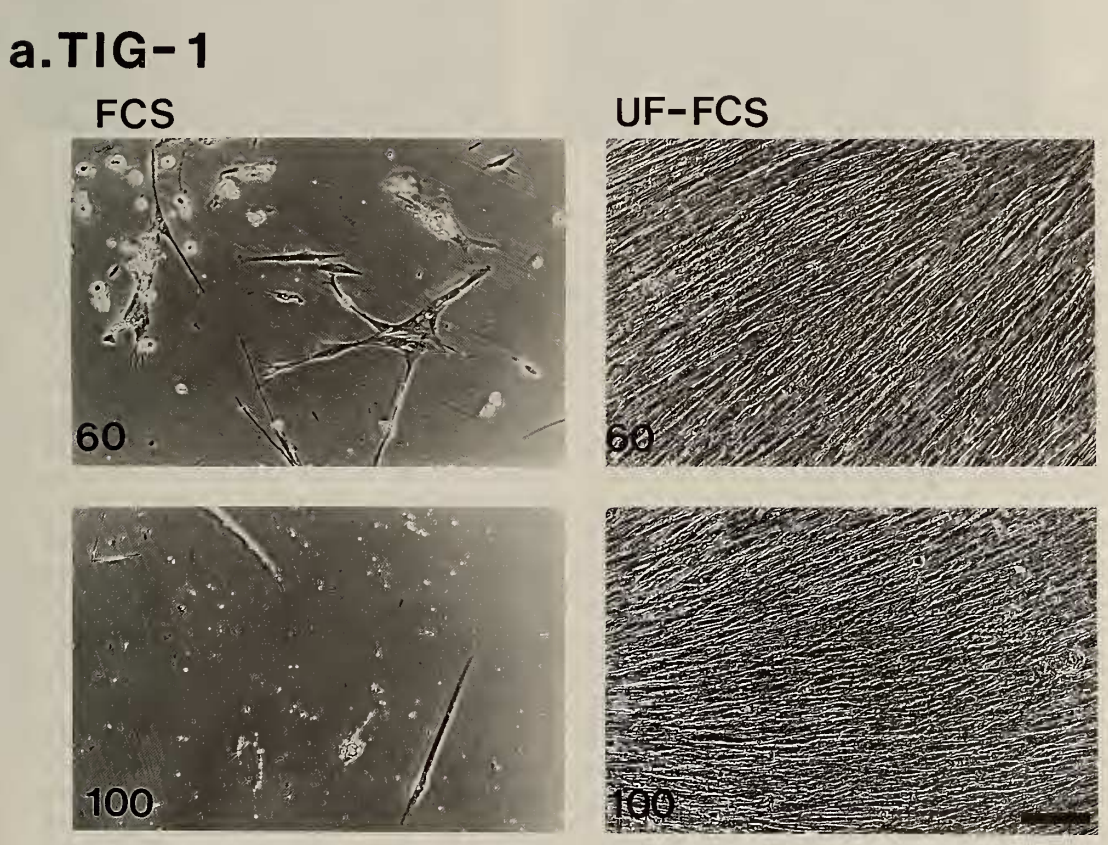

Fig. 4. a

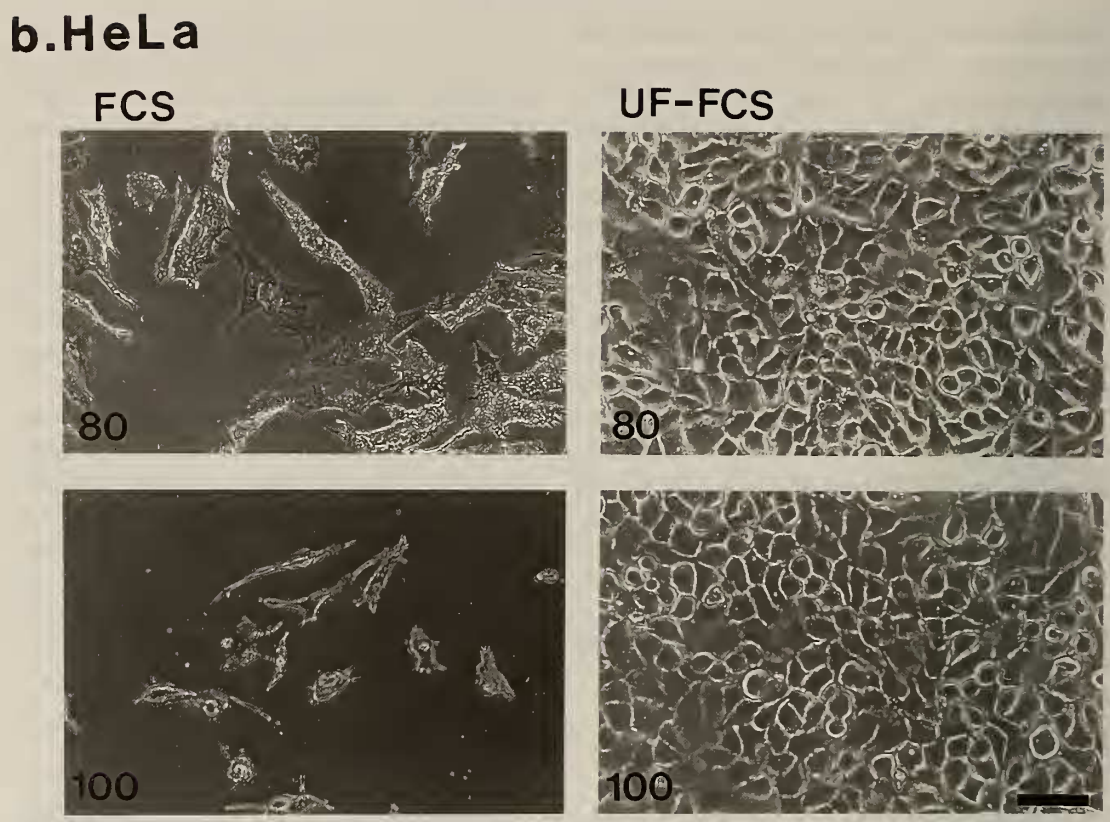

FIG. 4. b

FIG. 4. Effect of FBS on TIG-1 cells and HeLa cells. **a.** TIG-1 cells were seeded at 1.0×10^4 cells/well in a 24-well plate, and after 24 hr incubation at 37°C the medium was changed to MEM containing the indicated concentrations of FBS (left) or UF-FBS (right). Six days later photographs were taken. Bar=100 μm. **b.** Human epithelial carcinoma cells (HeLa) were seeded at 1.0×10^4 cells/well in a 24-well plate, and after 24 hr incubation at 37°C the medium was changed to MEM ontaining the indicated concentrations of FBS (left) or UF-FBS (right). Six days later photographs were taken. Bar=50 μm.

DISCUSSION

Based upon these results, it is likely that endogenous cytotoxic factors exist among the low molecular weight components of serum. FBS ultrafiltrate caused cell death, and the greater part of the cytotoxic factor(s) could pass through the YM2 filter (M.W. $<$1,000). The cytotoxicity, however, was slightly weaker than that of FBS. When the FBS fractions separated by ultrafiltration were recombined, the resultant mixture was more effective for cell growth but was no less cytotoxic than intact FBS. It may be hypothesized that there may exist a macro-molecular co-factor(s) that increases toxicity or amplifies the signal for cell death. It also possible that some of the toxic factor(s) was retained above ultrafilter by binding to macromolecules. Furthermore, we found that in some cases small amounts of high molecular-weight of proteins ($>$M.W. 3000) leaked through the membrane ($<10\ \mu$g/ml) (data not shown). It is also possible that low concentration of proteins play an important role in inducing cell death. At this point it is not possible to conclude with certainly that cell death was exclusively caused by low molecular weight factor(s). It is, however, clear that these factors are necessary for the cytotoxicity.

In answer to the question that the serum cytotoxicity might extraordinarily be produced only at blood coagulation and, if so, plasma might not exhibit such the toxicity, the present data of Fig. 7 that non of preparations of plasma differed from

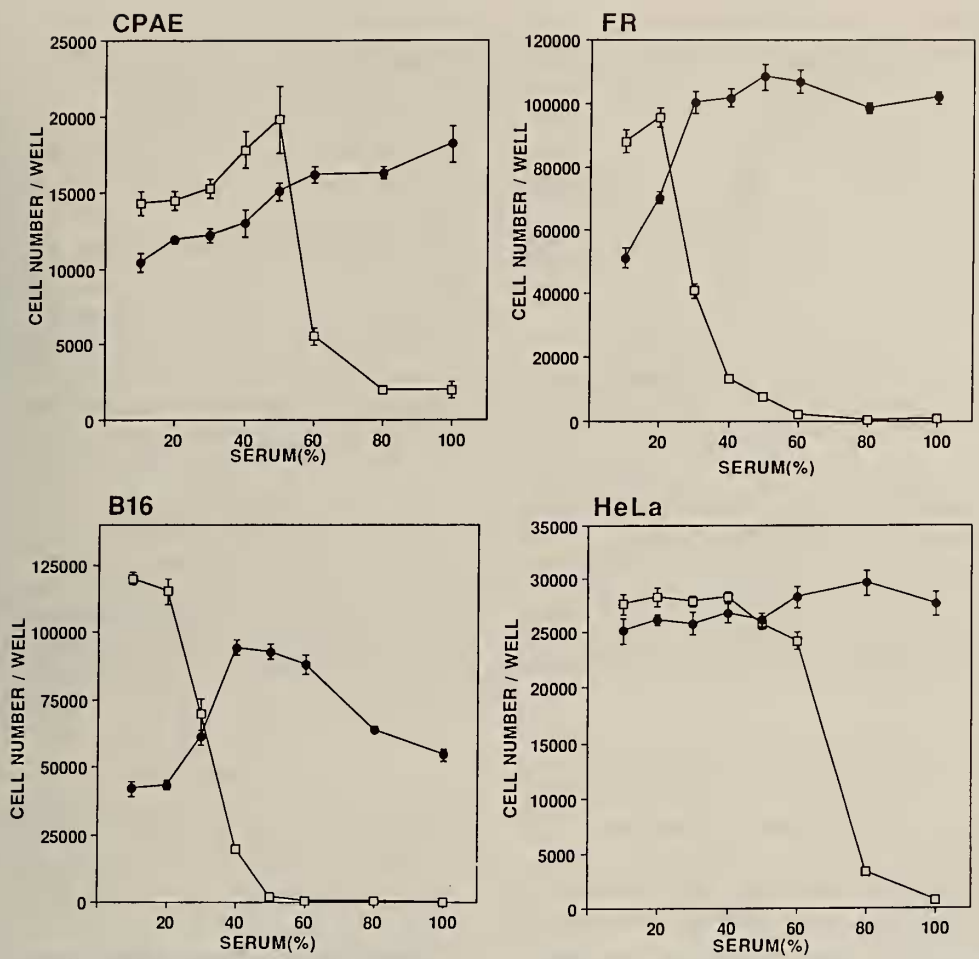

FIG. 5. Effect of serum concentration of growth of CPAE, FR, HeLa, and B16 cell lines. □: FBS, ●: UF-FBS. Cells were inoculated at the following densities in 96-well plates; CPAE 3×10^3/well, FR 4×10^3/well, HeLa 3×10^3/well, B16 6×10^3/well. After 24 hr incubation at 37°C the medium was changed to MEM containing the indicated concentrations of FBS. Six days later, cell number was determined with a Coulter Counter.

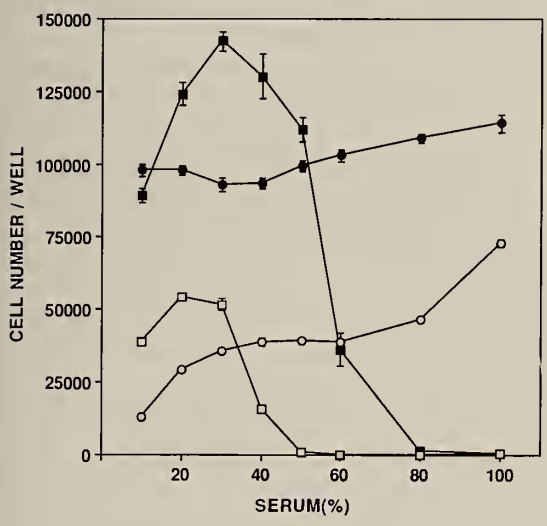

FIG. 6. Serum effect on the primary culture of a rat whole-embryo. A primary culture of a rat whole embryo cells was seeded 6.0×10^3 (□: FBS, ○: UF-FBS) and 6.0×10^4 (■: FBS, ●: UF-FBS) cells/well in a 24-well plate. After 24 hr incubation at 37°C the medium was changed to MEM containing the indicated concentrations of FBS. Six days later, cell number was determined with a Coulter Counter.

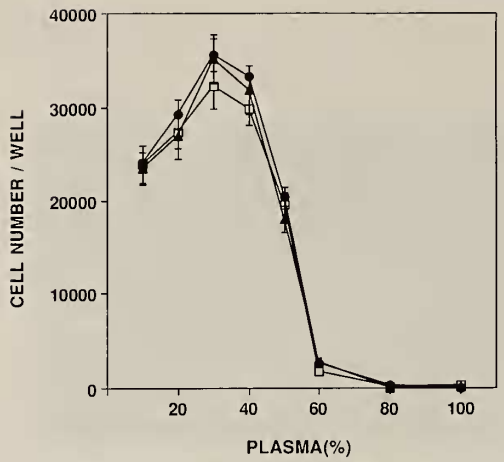

FIG. 7. Effect of plasma concentration on growth of TIG-1 fibroblast. ●: crude plasma, □: thrombin-treated plasma, ▲: incubated plasma. Human fetal lung fibroblast TIG-1 cells were seeded at 5.0×10^3 cells/well in a 96-well plate, and after 24 hr incubation at 37°C medium was changed to MEM containing the indicated concentrations of bovine plasma. Six days later, cell number was determined with a Coulter Counte.

FBS suggested the cytotoxicity existed normally in blood.

As to the question of why the toxic factor(s) do not cause cell death *in vivo*, one explanation may be that the cells are not exposed to the same high concentrations of these factors that are present *in vitro*. Most tissues are protected by an endothelial barrier and are not exposed to blood directly. If so, endothelial cells *in vivo* may be less sensitive to FBS cytotoxicity. CPAE bovine aorta endothelial cells showed cell death in FBS, however (Fig. 4B), it may be that the CPAE cell line lost this property during long-term culture. In a preliminary experiment we have that bovine aorta endothelial cells in the primary culture survived and even grew in 100% FBS (data not shown). By contrast, rat whole-embryo primary cultures did not grow and almost all the cells died in FBS (Fig. 6). It seems that most cell types except vascular endothelial cells can not survive in FBS *in vitro*.

The molecular identity of the cytotoxic factor is still unclear, but cells cultured in either Hank's balanced salt solution and Dulbecco's PBS did not showed cell death (data not shown). This suggests that cell death is not caused by simple starvation, nutritional imbalance, or a defect of physicochemical conditions of culture.

In vivo, all normal cell growth is well controlled, but once cells are transplanted *in vitro*, they start to rapidly proliferate in serum containing media. Vigorous cell growth may reflect the response at a site of injury. Therefore it is possible that cytotoxic factor(s) is a negative growth regulator or an inducer of cell death in tissue injured *in vivo*.

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REFERENCES

1 Baker LE, Carrel A (1925) Lipids as the growth-inhibiting factor in serum. J Exp Med 42: 143–154

2 Carrel A, Ebeling AH (1921) Age and multiplication of fibroblasts. J Exp Med 34: 143–154

3 Evans JV, Jackson LJ, Andersen WF, Mitchell JT (1967) Chromosomal characteristic and neoplastic transformation of C3H mouse embryo cells *in vitro* in horse and fetal calf serum. J Nat Cancer Ins 38: 761–769

4 Ham RG, Mckeehan WL (1978) Nutritional requirement for clonal growth of nontransformed cell. Japan Sci Soc Press Tokyo, pp 63–115

5 Hayashi I, Sato G (1976) Replacement of serum by hormones permits growth of cells in defined medium. Nature 259: 132–134

6 Sato G (1981) A working hypothsis for cell culture Hormones and cell regulation. INSERM Europ Symp, Elsevier/North-Holland Biomedical Press, Amsterdam, 5: 1–13